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Litter of the invasive shrub *Rhododendron ponticum* (Ericaceae) modifies the decomposition rate of native UK woodland litter.

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Abstract

Invasive alien plants are a worldwide problem, causing substantial damage to biodiversity as well as economies. Recent studies suggest invasive plants may also alter fundamental ecosystem processes such as nutrient and carbon cycling in soil by depositing chemically distinct leaf litter. Here, we used laboratory microcosms to test whether the chemical properties of *Rhododendron ponticum* litter, an invasive shrub in Britain, lead to slower decomposition than that of native (or naturalised) species with labile litter (*Acer pseudoplatanus* and *Fraxinus excelsior*), but not relative to the recalcitrant litter of *Quercus petraea*. Leading from this, we hypothesised that the labile native litter decomposition rate is reduced when mixed with *R. ponticum* litter in non-additive responses, with the strength of these responses increasing with the proportion of *R. ponticum* in litter mixes (25%, 50% and 75% *R. ponticum*). Over the incubation period, the decomposition (measured as the microbial respiration rate) of unmixed *R. ponticum* litter was significantly lower than that of *A. pseudoplatanus* and *F. excelsior*, but not *Q. petraea*. When mixed with *R. ponticum* (50%), *F. excelsior* litter decomposition was slowed, whilst no effect was seen for *Q. petraea*. However, *A. pseudoplatanus* litter decomposition was enhanced, contrary to expectation. The strength of the non-additive decomposition responses did not vary with different proportions of *R. ponticum* to the other species, with only the 50% mixtures showing significant non-additive respiration rates. Litter chemical properties were highly associated with decomposition rates, with both phenolic content and C:N ratio negatively correlated with microbial respiration. To test the influence of phenolics on litter decomposition, leachates of *R. ponticum* litter with phenolics present or removed (via activated carbon) were added to microcosms containing the native species litter. Microbial respiration in *F. excelsior* microcosms was lower when *R. ponticum* leachate contained phenolics. For *A. pseudoplatanus* and *Q. petraea* litter, no effect of leachate treatment was observed. Our results show that invasive litter chemistry can alter the decomposition of native litter, with the impact varying between species. Altered decomposition rates could cause plant-

soil feedbacks, leading to altered soil nutrient concentrations. The novel soil conditions may favour the invader, increasing its dominance, whilst negatively influencing native species possessing greater nutrient demands.

Keywords: Invasive; litter; decomposition; non-additive; phenolic; ecosystem; soil.

1. Introduction

Nutrient cycling is an essential ecosystem service and decomposition is a key component in this process (Delgado-Baquerizo et al., 2017). Decomposition involves soil organisms breaking down organic matter, releasing nutrients as soluble inorganic nutrients (Delgado-Baquerizo et al., 2017; Gartner and Cardon, 2004). As a result, organic matter decomposition influences nutrient availability, therefore influencing the vegetation community that can inhabit the soil (Van der Putten et al., 2013).

The rate of decomposition is determined by plant litter quality, along with the soil microbial community and physicochemical properties (Jewell et al., 2015). At the ecosystem level, litter chemistry is the main influence on decomposition (Aerts, 1999; Strickland et al., 2009). Plants adapted to low-nutrient environments, typically produce litter with high C:N ratios and polyphenol contents which protect leaf tissues by deterring herbivory (Aerts, 1999; Hobbie, 1992; Kuiters, 1990). Many phenolic compounds however inhibit decomposition and nutrient cycling, by suppressing microbial activity and complexing with proteins (Fanin et al., 2014; Horner et al., 1988). The resulting slow decomposition of the recalcitrant litter, leads to low soil concentrations of inorganic nitrogen, the main source of nitrogen for the majority of plant species (DeLuca et al., 2013; Hobbie, 1992; Michelsen et al., 1996; Nielsen et al., 2009). By lowering nutrient availability in such plant-soil feedbacks, a species with low nutrient demands may enhance its competitiveness and become dominant (Van der Putten et al., 2013). Ericaceous species in particular are known to influence soil conditions via litter decomposition, leading to their dominance in low nutrient environments where inorganic nitrogen does not accumulate in sufficient

66 concentrations for species with higher nutrient demands (Aerts, 1999; DeLuca et al., 2013; Michelsen et
67 al., 1998; Wurzburger and Hendrick, 2009).

68 The litter of one species rarely occurs alone in the natural environment; litter layers usually contain a
69 mixture of different species which decompose together (Gartner and Cardon, 2004). Since the 1980s
70 there have been several studies comparing the decomposition rate of litter mixes with expected values
71 calculated from the decomposition rates of the individual component species. Gartner and Cardon
72 (2004) reviewed these studies, finding non-additive decomposition, that is responses which were
73 different to calculated expected values, in many of the studies reviewed. Non-additive decomposition
74 may be explained by many factors. Litter chemistry is important, as some species release nutrients or
75 secondary metabolites as they decompose. Nutrient release may accelerate decomposition in more
76 recalcitrant, adjacent material, a synergistic response (Hector et al., 2000; Salamanca et al., 1998). On
77 the other hand, the inhibitory properties of leached phenolic compounds may cause antagonistic
78 responses, where the decomposition rate of more labile adjacent litter is slowed (Hector et al., 2000;
79 McArthur et al., 1994). Additionally, compounds leaching from litter may induce shifts in the soil
80 microbial community, leading to such responses (Hector et al., 2000; Wardle et al., 1998). Finally, the
81 greater diversity of habitats litter mixtures provide for decomposer organisms may also lead to
82 synergistic responses (Hansen and Coleman, 1998; McArthur et al., 1994; Salamanca et al., 1998).

83 Plant invasions are often associated with non-additive decomposition (Gartner and Cardon, 2004), with
84 the strength of these interactions increasing with the proportion of invasive litter in the mixtures
85 (Elgersma and Ehrenfeld, 2011; Hickman et al., 2013). The majority of studies have found invasive litter
86 to accelerate native litter decomposition (e.g. Schuster and Dukes, 2014), with relatively few studies
87 finding antagonistic decomposition following plant invasions (Hickman et al., 2013; Zhang et al., 2014).
88 In one of the rare studies to find antagonistic responses following litter mixing, Rosemond et al. (2010)
89 observed slower decomposition when *Rhododendron maximum* L. litter was mixed with *Acer rubrum* L.

and *Liriodendron tulipifera* L. in a freshwater stream. The inhibited decomposition was attributed to the high C:N ratio of *R. maximum* relative to the other two species, as the effect was alleviated where nitrogen was added to the water (Rosemond et al., 2010).

Altering ecosystem processes in a similar way to *R. maximum* via non-additive decomposition may be a driver behind the success of the related *Rhododendron ponticum* L.. Following its introduction to Britain from Spain in 1763 as an ornamental shrub, *R. ponticum* has become a highly damaging invader of native habitats (Cross, 1975). It is particularly problematic in broadleaved woodlands, where the dense shade cast by its canopy prevents the regeneration of tree species such as *Fraxinus excelsior* L. and *Quercus petraea* Matt. (Liebl.) (Cross, 1975; Jackson, 2008; Peterken, 2001). In addition to the direct effect of canopy shading, *Rhododendron* spp. are known to deposit recalcitrant acidic litter, which is high in polyphenols and low in nitrogen (Monk et al., 2014; Wurzburger and Hendrick, 2007). Its slow decomposition leads to an accumulation of a thick litter layer and the formation of infertile soils which may disadvantage competing species with higher nutrient requirements (Monk et al., 2014; Plocher and Carvell, 1987). Therefore, the chemical properties of *R. ponticum* litter may suppress the decomposition of native tree species in invaded habitats (Nilsen et al., 1999; Rosemond et al., 2010; Wurzburger and Hendrick, 2009). Such non-additive responses have significant implications for vegetation communities post-invasion, as they influence nutrient availability (Richards et al., 2010), potentially shifting the natural balance of an ecosystem towards an altered state (Suseela et al., 2016).

This investigation aims to determine whether the chemical properties of invasive *R. ponticum* litter contribute towards non-additive decomposition when mixed with three native (or naturalised) tree species commonly found in the invaded broadleaved woodlands; namely *Acer pseudoplatanus* L., *F. excelsior* and *Q. petraea*. Using microcosm assays, we test four hypotheses. Firstly, that initial litters vary in their phenolic compound and nutrient content between species. Secondly, that due to its chemical properties which are supposed to inhibit decomposition, the litter of *R. ponticum* decomposes more

slowly than the more labile litter of *A. pseudoplatanus* and *F. excelsior*, but similar to the recalcitrant litter of *Q. petraea*. Decomposition was monitored as microbial respiration and as dissolved organic carbon leached from the microcosms at various timepoints during the incubation. Thirdly, that due to compounds leaching from the polyphenol-rich *R. ponticum* litter, mixing *R. ponticum* litter with labile native litter in microcosms produces antagonistic decomposition responses, whilst having no effect on more recalcitrant litter. To further test the role of phenolic compounds in native litter decomposition, leachates from decomposing *R. ponticum* litter were added to single species microcosms containing one of the native species. Finally, we hypothesise that the strength of any non-additive responses increases with increasing proportions of *R. ponticum* in the litter mixes, due to the leaching of more phenolic compounds. To interpret the results, we analysed initial litter samples for chemical properties that influence decomposition (carbon content, nitrogen content, C:N ratio, phenolic content and pH).

2. Materials and methods

2.1. Sample collection and preparation

During October 2017, freshly senesced, undecomposed leaf litter samples showing autumnal colours (Cornelissen, 1996) were collected for *R. ponticum* and three native or naturalised (referred to as native from here on) tree species from a broadleaved woodland in Ceredigion, Wales (52°25'11"N 4°4'12"W). *A. pseudoplatanus*, *F. excelsior* and *Q. petraea* were selected as tree species as they are commonly found in native broadleaved woodlands, a habitat threatened by *R. ponticum* invasion (Peterken, 2001), and due to the varying degrees of decomposability of their litters (Slade and Riutta, 2012). All three native species coexisted in the woodland invaded by *R. ponticum*. Litter samples were air dried to constant weight at 25 ^{o}C for 8 days, then homogenised using a benchtop ball mill (Retsh MM200, Haan, Germany) (particle size <500 µm). The low drying temperature was selected to minimise the degradation of secondary compounds which influence decomposition rates (Hoorens et al., 2003). Samples were milled following the microcosm method employed by Strickland et al. (2009) to remove

the effect of litter physical properties, in order to focus on the influence of litter chemical properties on non-additive responses in decomposition. Following this, 13 different litter treatments were prepared, which covered all possible combinations with *R. ponticum*. These consisted of unmixed litter for each individual species (100%), as well as mixtures of each native species (*A. pseudoplatanus*, *F. excelsior* or *Q. petraea*) with varying mass proportions of *R. ponticum* (25%, 50% and 75% *R. ponticum*) to replicate different litter layers at the interface with competing species.

2.2. Litter chemistry

Subsamples of initial litter for each of the four studied species were analysed for chemical properties that influence decomposition (Table 1). Litter carbon and nitrogen content, and C:N ratios were measured by igniting 200 mg of material in a Vario MAX cube analyser (Elementar, Langenselbold, Germany). Total phenolic content was measured using the Folin-Ciocalteu method (Makkar et al., 1996). Briefly, phenolics were extracted by shaking 30 mg of sample in 2 mL of 90% methanol for 10 minutes. The suspension was then centrifuged for 10 minutes at 13,000 rpm before decanting the supernatant. The extraction process was repeated by resuspending the pellet in 2 mL 90% methanol, resulting in 4 mL of extract solution. Absorbance was measured at 725 nm using a gallic acid calibration curve. Litter pH was analysed by suspending 1 g of ground litter in 5 mL of distilled water, before measuring with a pH meter (Fisherbrand Hydrus 500, Loughborough, UK). Total soluble organic carbon content of the microcosm leachates was measured using a carbon analyser (Thermalox TOC-TN, Analytical Sciences Ltd., Cambridge, UK).

Table 1: Initial litter chemical properties of the four studied species included in the study (± standard error) (n = 7). Total phenolic content was measured as gallic acid equivalent (GAE). Common letters denote statistically non-significant differences between the means ($P < 0.05$) following analyses in GLMs (further discussed in the results section).

Species	C (%)	N (%)	C:N	pH	Total phenolics (µg GAE mg^{-1} dry weight)
R. ponticum	46.06 ± 0.05 a	1.01 ± 0.01 a	45.56 ± 0.34 a	5.26 ± 0.01 a	98.18 ± 1.15 a
A. pseudoplatanus	45.68 ± 0.07 b	1.20 ± 0.01 b	38.01 ± 0.28 b	5.52 ± 0.03 b	68.24 ± 0.67 b
F. excelsior	44.42 ± 0.04 c	2.14 ± 0.01 c	20.80 ± 0.11 c	5.26 ± 0.02 a	34.77 ± 0.52 c
Q. petraea	47.15 ± 0.05 d	1.04 ± 0.01 a	45.58 ± 0.39 a	4.57 ± 0.04 c	126.99 ± 0.65 d

161

162 *2.3. Litter decomposition microcosms*

163 Decomposition microcosms were constructed based on previous studies (Jones et al., 2016; Wardle et
164 al., 2009). For each microcosm, 10 g of sterile acid-washed sand (250-500 µm) was placed in 50 mL
165 syringe barrels (BD Plastipak, Madrid, Spain), which were held upright in a randomised design in a rack,
166 their tips sealed with Suba Seals (no. 9). The acid-washed sand provided an inert media to place 200 mg
167 of each of the 13 litter combinations (n = 7 per treatment).

168 Litter decomposition was initiated by adding 3 mL of a homogeneous microbial inoculant solution,
169 common to all treatments. This solution was extracted based on the methods of Jones et al. (2016) and
170 Gehrke et al. (1995), where 50 g of recently senesced native litter, showing autumnal colours and
171 collected from the same sampling site, was suspended in 1 L of distilled water for eight hours, a ratio
172 representative of typical rainfall and litter cover in the area, before filtering twice through Whatman no.
173 1 filter paper (Whatman Paper Ltd., Maidstone, UK). The litter used to make the inoculant solution
174 contained equal amounts of all three native species, as a microbial community's "perception" of litter
175 quality is determined by the parent plant community, thus avoiding bias between native species
176 (Strickland et al., 2009). *R. ponticum* litter was not included, resulting in a native microbial inoculant that
177 had not yet been affected by its invasion, as the main aim of the study was to investigate how the

178 introduction of invasive litter influences native litter decomposition. Syringe barrels were then sealed
179 with Suba Seals (no. 57) to prevent water loss and incubated in a darkened growth chamber for 12
180 weeks at 22 °C, a temperature commonly used in such controlled microcosm experiments on litter
181 decomposition (e.g. Jones et al., 2016; Wardle et al., 2009). The upper seals were removed for two
182 minutes at seven-day intervals during the incubation to renew the air within the chambers and prevent
183 anoxic conditions, following the method of Jones et al. (2016).

184 At six fortnightly timepoints during the incubation, microbial respiration within the chambers was
185 measured using a method based on that used by Gehrke et al. (1995). Briefly, this involved removing the
186 upper seal, before flushing the chambers with air to lower the CO_2 concentration to ambient levels. The
187 initial CO_2 concentration within the microcosms was measured by sampling 5 mL of air with a syringe,
188 which was directly injected into an infra-red gas analyser (IRGA) (EGM-4, PP-systems, USA). The upper
189 seal was then replaced, before a second 5 mL sample of air was taken after two minutes, using a needle
190 which penetrated the Suba Seal septum. The air sample was subsequently injected into the IRGA, which
191 measured the spike in CO_2 concentration. Respiration, measured as the rate of CO_2 accumulation, was
192 calculated using the below equation (1), based on information given in the PP Systems soil respiration
193 chamber manual (2005):

194 $$Accumulation\ rate = \frac{F-I}{t} \times \frac{P}{1000} \times \frac{273}{273+T} \times \frac{44.01}{22.41} \times \frac{V}{A} \qquad (1)$$

195 where F = final CO_2 concentration, I = initial CO_2 concentration, t = time in seconds, P = atmospheric
196 pressure, T = a constant temperature of 22°C, V = chamber volume and A = chamber surface area.
197 Respiration rate measurements were subsequently converted to g of $CO_2\ m^{-2}\ h^{-1}$ for analyses and
198 presentation, as this is the most commonly used form for field measurements.

199 Following the respiration measurements at each timepoint, leachates were collected from the
200 microcosms based on the method of Jones et al. (2016). This was done by adding 4 mL of distilled water

to each tube, before applying pressure with a syringe plunger to extract 4 mL of leachate from the tips. Leachates were stored at -80°C prior to analysing for total organic carbon.

To investigate whether litter mixing resulted in non-additive responses, observed values were compared to expected values for each litter mix, as in previous studies reviewed by Lecerf et al. (2011). Expected values for 50% *R. ponticum* – 50% native mixes were calculated as:

$$Expected\ value = \frac{(x+y)}{2} \qquad (2)$$

where x = observed value for *R. ponticum* and y = observed value for native species. Equation (2) was adapted to equation (3) for litter mixtures which were 75% *R. ponticum* and 25% native, and equation (4) for 25% *R. ponticum* and 75% native litter mixtures.

$$Expected\ value = \frac{(3x+y)}{4} \qquad (3)$$

$$Expected\ value = \frac{(x+3y)}{4} \qquad (4)$$

The strength of non-additive responses following litter mixing was estimated using an equation (5) based on Hoorens et al. (2003):

$$Non-additive\ response\ strength = \left(\frac{O}{E}\right) - 1 \qquad (5)$$

where O = the observed value for mean respiration and E = the expected value mean respiration, calculated as described above. The stronger the response, the greater the deviation from 0. Where there were synergistic responses, the strength values were positive, whilst the values were negative for antagonistic responses.

Microcosm contents were removed after 12 weeks and oven-dried at 40°C to constant weight. Litter was separated from the sand by sieving, and then stored at -80 °C prior to chemical analyses.

221 *2.4. R. ponticum leachate addition experiment*

222 A follow-up microcosm experiment was conducted to investigate the influence of compounds leaching
223 from decomposing *R. ponticum* litter on microbial respiration in microcosms containing *A.*
224 *pseudoplatanus*, *F. excelsior* or *Q. petraea* litter. To collect decomposing *R. ponticum* litter leachate,
225 microcosms containing *R. ponticum* litter were incubated using the method described above. After two
226 weeks, 2 mL of distilled water was added to each microcosm and leachate was collected as previously
227 described. The collected leachate was split into two aliquots; one was left unaltered, whilst the other
228 was treated with activated carbon, which lowered total phenolic content by over 97% (Table 1S).
229 Activated carbon was added to leachate at 50 g L^{-1}, and both batches were then stirred for 5 hours
230 (Mukherjee et al., 2007). The leachates were then centrifuged at 13,000 rpm for 5 minutes to remove
231 solids, before the supernatant was transferred to clean bottles.

232 Microcosms containing either *A. pseudoplatanus*, *F. excelsior* or *Q. petraea* litter were subsequently
233 prepared as previously described. Decomposition in these microcosms was initiated with either the
234 unaltered leachate, activated carbon treated leachate or distilled water (n = 7). Microbial respiration in
235 these native species microcosms was measured after one, five, ten and 15 days of incubation, using the
236 method previously described.

237 *2.5. Statistical analyses*

238 All statistical analyses were conducted using R programming software version 3.5.3 (R Development
239 Core Team, 2017). Generalised linear models (GLMs) were used to compare initial litter chemical
240 properties between species (phenolic content, C:N and pH). Generalized linear mixed models (GLMMs)
241 were used for repeated measures of microbial respiration and leached organic carbon over the duration
242 of the incubation, using the *lme4* package and the *multcomp* package for subsequent pairwise
243 comparison. GLMs or independent sample t-tests were used to analyse data within individual
244 timepoints. Pearson's product moment correlation tests investigated the relationship between microbial

245 respiration and leached carbon, as well as between litter chemical properties and the cumulative
246 respired CO_2 and leached organic carbon, calculated using the area under the curves as in Strickland et
247 al. (2009).

248 **3. Results**

249 *3.1. Initial litter chemistry*

250 The C:N ratio of *R. ponticum* litter was significantly higher than both *F. excelsior* and *A. pseudoplatanus*
251 ($P < 0.001$), but there was no significant difference relative to *Q. petraea* ($P = 0.990$) (Table 1). Phenolic
252 compound concentration in *R. ponticum* litter was significantly higher than in *A. pseudoplatanus* and *F.*
253 *excelsior* litter ($P < 0.001$), whilst *Q. petraea* litter had significantly higher concentrations than all three
254 other species ($P < 0.001$). *R. ponticum* litter pH was significantly lower than that of *A. pseudoplatanus* (P
255 < 0.001), but not *F. excelsior* ($P = 0.390$). *Q. petraea* litter pH was significantly lower than all other litters
256 ($P < 0.001$).

257 *3.2. Single species litter microcosms*

258 Unmixed litter samples of the four species were compared to investigate decomposition between
259 species. Repeated measures analysis showed respiration (g CO_2 m^{-2} h^{-1}) in microcosms containing *R.*
260 *ponticum* litter was significantly lower than in those containing *A. pseudoplatanus* ($P = 0.026$) and *F.*
261 *excelsior* ($P < 0.001$) litter, but not significantly lower relative to *Q. petraea* litter ($P = 0.802$) (Figure 1).
262 Following on from this, microbial respiration in these microcosms was compared within specific
263 timepoints, which revealed temporal variation. Microbial respiration in microcosms containing *A.*
264 *pseudoplatanus* was significantly higher than for *R. ponticum* microcosms only at six ($P = 0.047$), eight (P
265 < 0.001) and ten weeks into the incubation ($P < 0.001$). There was no significant difference between *R.*
266 *ponticum* and *F. excelsior* microcosm respiration two weeks into the incubation ($P > 0.05$). Respiration
267 was significantly higher during every subsequent timepoint in the *F. excelsior* microcosms relative to *R.*

ponticum (four, six, eight and ten weeks: $P < 0.001$, 12 weeks: $P < 0.05$), while *R. ponticum* and *Q. petraea* microcosm respiration did not significantly differ at any of these time points ($P > 0.05$). Cumulative respired CO_2, measured as the area beneath the microbial respiration curve, was strongly and negatively correlated with both litter C:N ratio and phenolic content ($P < 0.001$, $R^2 = -0.89$ and $P < 0.001$, $R^2 = -0.84$ respectively), whilst it also showed a weak, positive correlation with litter pH ($P = 0.034$, $R^2 = 0.4$).

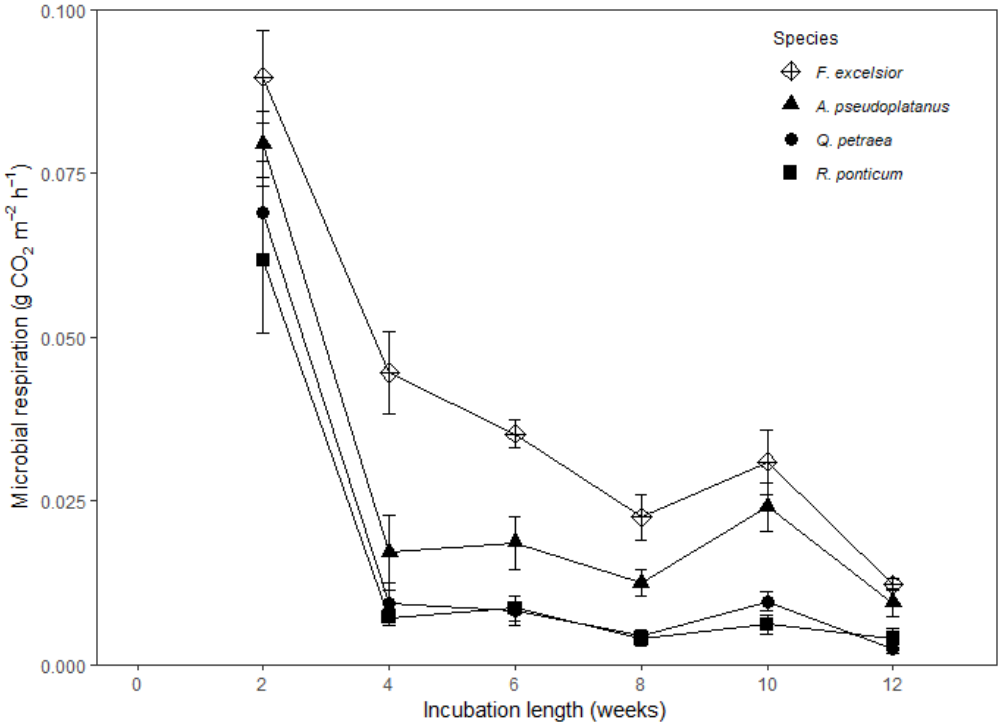

Figure 1: Mean microbial respiration (g CO_2 m^{-2} h^{-1}) for the microcosms containing the unmixed litter of *R. ponticum*, *A. pseudoplatanus*, *F. excelsior* and *Q. petraea* (n = 7). Error bars represent the standard error.

Microbial respiration in the single species microcosms was significantly correlated to their leached carbon concentrations ($P < 0.001$, $R^2 = 0.73$). Repeated measures analysis showed leachates from *F. excelsior* had significantly higher carbon concentrations than leachates from *R. ponticum*, *A. pseudoplatanus* and *Q. petraea* ($P < 0.001$) (Figure 2). No significant differences were observed between

the other species (P > 0.05). Higher dissolved organic carbon concentrations for *F. excelsior* leachates were also observed within timepoints; two weeks into the incubation, *F. excelsior* microcosm leachate carbon content was significantly higher than that of *A. pseudoplatanus* and *Q. petraea* (P < 0.001), but not *R. ponticum* (P = 0.383). After four weeks of incubation, the leachate carbon content of *F. excelsior* was significantly higher than all three other species (P < 0.001). No differences between any of the species were observed during weeks six and eight (P > 0.05). However, during weeks ten and 12, the carbon content of *F. excelsior* leachate was again significantly higher than all other species (P < 0.001). Cumulative leached carbon, measured as the area beneath the leachate carbon concentration curve, was negatively correlated with initial litter C:N (P < 0.001, R^2 = -0.74) and phenolic content (P < 0.001, R^2 = - 0.61), however there was no relationship with litter pH (P = 0.489, R^2 = 0.14).

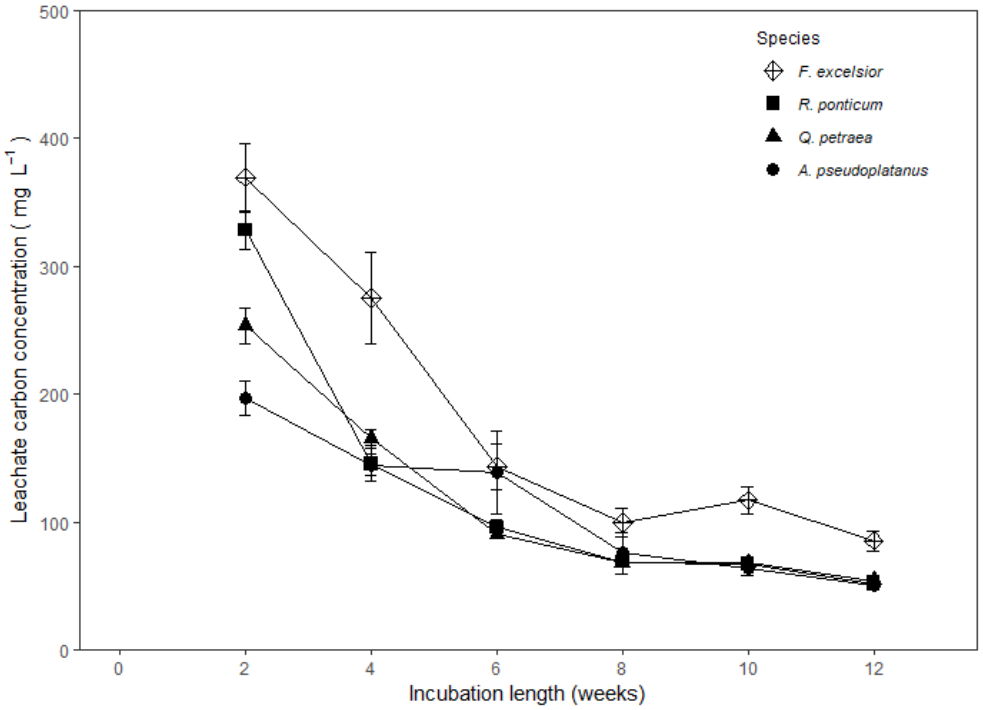

Figure 2: Mean leachate total organic carbon concentration (mg L^{-1}) for the microcosms containing the unmixed litter of *R. ponticum*, *A. pseudoplatanus*, *F. excelsior* and *Q. petraea* (n = 7). Error bars represent the standard error.

3.3. Non-additive decomposition microcosm experiment

Non-additive microbial respiration was only observed in 50% mixes with *R. ponticum*, being synergistic for *A. pseudoplatanus* ($P < 0.001$) and antagonistic for *F. excelsior* ($P = 0.025$) (Figure 3). No non-additive interactions were observed for these species when mixed at other percentages (25% or 75%), or in any litter mix with *Q. petraea* ($P > 0.05$). When comparing the 50% microcosms within timepoints (Figure 3), significant differences ($P < 0.05$) between observed and expected values were seen for *A. pseudoplatanus* only during the second week. For *F. excelsior*, observed and expected values differed only during weeks four, six and eight ($P < 0.05$).

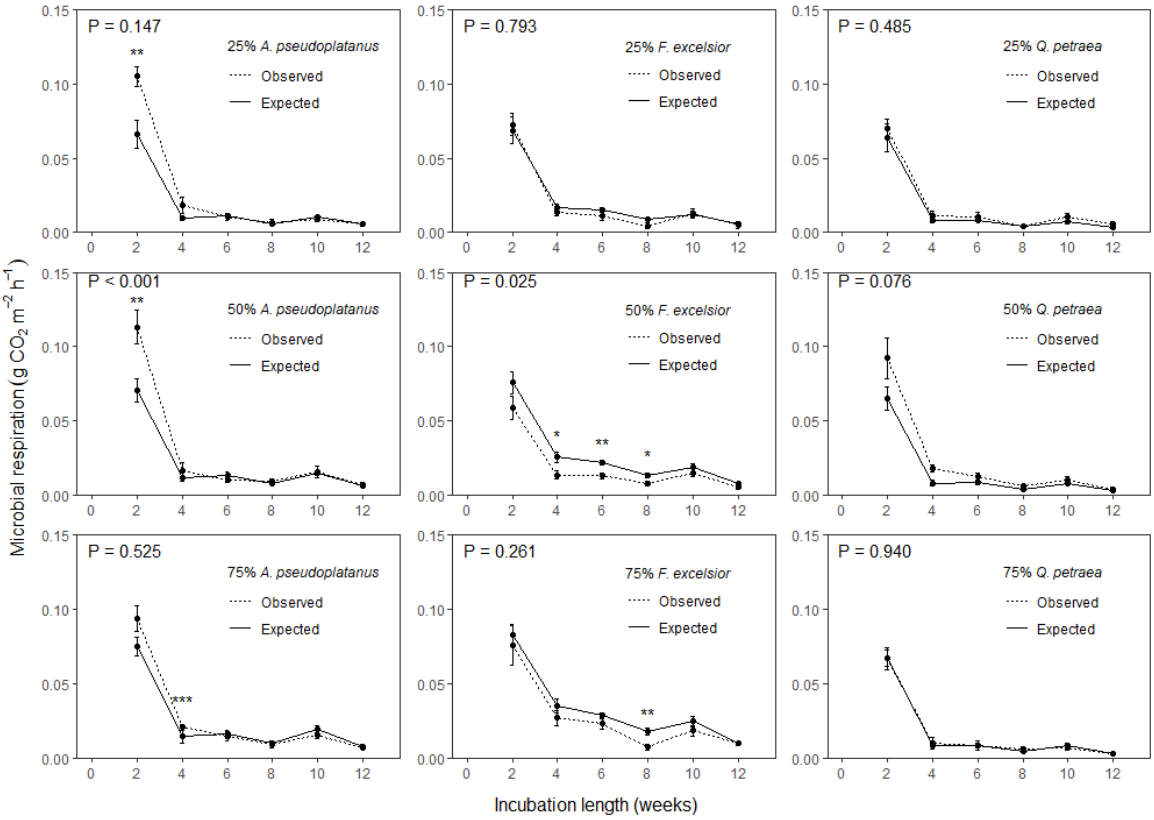

Figure 3: Expected and observed microbial respiration data (g CO_2 m^{-2} h^{-1}) for the microcosms containing *A. pseudoplatanus*, *F. excelsior* or *Q. petraea* litter, mixed with *R. ponticum* litter at different proportions (n = 7). Expected values were calculated from the microbial respiration of the individual component species, using the equations described in the methods section. Error bars represent standard error.

Overall significance between observed and expected values was tested using GLMMs, with the P value displayed in the top-left corner of each panel. T-tests were used to analyse data within timepoints, with significance denoted above the points (* P < 0.05; ** P < 0.01; *** P < 0.001).

Antagonistic non-additive responses in leached carbon concentrations were observed for both *A. pseudoplatanus* and *F. excelsior* when mixed with *R. ponticum* at 50% (P < 0.001) (Figure 4). Antagonistic responses were also observed for *A. pseudoplatanus* when mixed with 25% and 75% *R. ponticum* litter (P = 0.027 and P < 0.001 respectively). No non-additive interactions were observed for the 25% and 75% *F. excelsior* litter mixtures (P = 0.340 and P = 0.068 respectively), or for any mixture containing *Q. petraea* litter (P > 0.05).

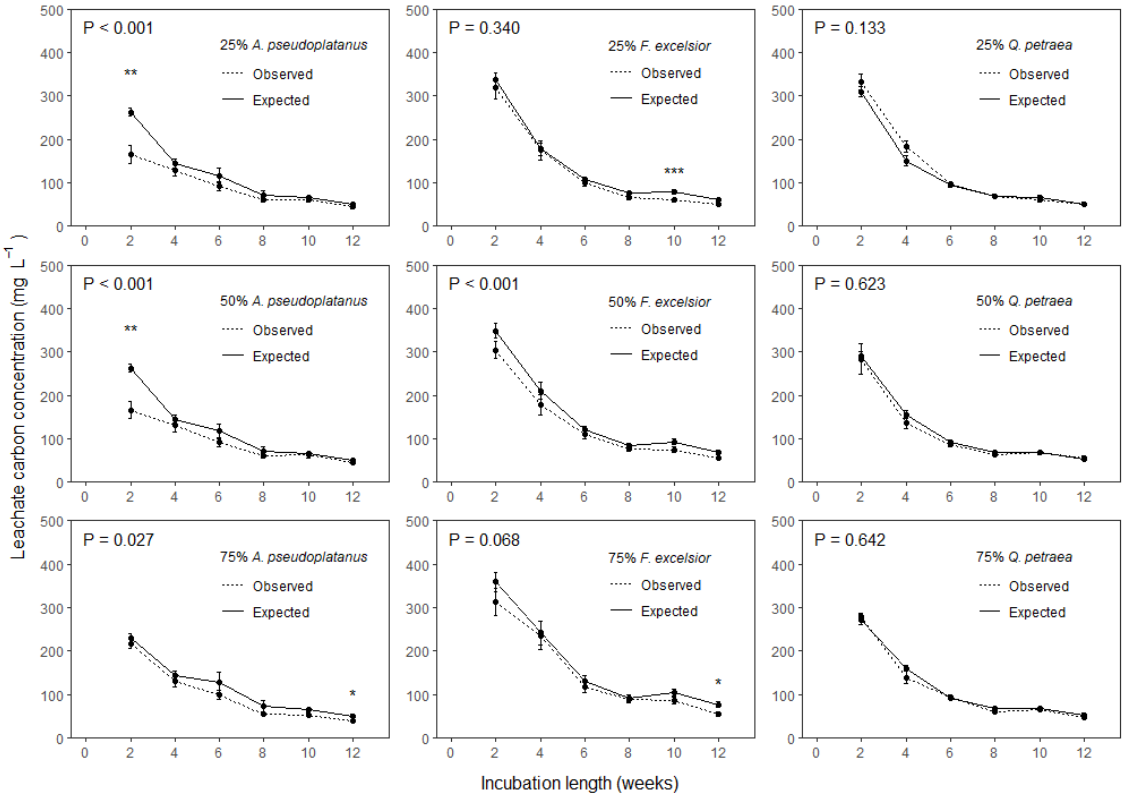

Figure 4: Expected and observed leachate total organic carbon concentration (mg L^{-1}) for the microcosms containing *A. pseudoplatanus*, *F. excelsior* or *Q. petraea* litter, mixed with *R. ponticum* litter at different proportions (n = 7). Expected values were calculated from the microbial respiration of the

individual component species, using the equations described in the methods section. Error bars represent standard error. Overall significance between observed and expected values was tested using GLMMs, with the P value displayed in the top-left corner of each panel. T-tests were used to analyse data within timepoints, with significance denoted above the points (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Non-additive response strength was calculated based on Hoorens et al. (2003) (see equation five in the materials and methods) (Table 2). The proportion of *R. ponticum* included in the litter mixture had no impact on the response strength for neither *A. pseudoplatanus*, *F. excelsior* nor *Q. petraea* ($P = 0.468$, $P = 0.386$ and $P = 0.179$ respectively). Furthermore, in a two factor GLM (species x litter proportion), the proportion of *R. ponticum* litter had no impact on response strength ($P = 0.209$), however species had a significant effect ($P = 0.001$). No significant interaction was observed between these two factors ($P = 0.510$).

Table 2: The strength of the non-additive response ($\pm$ standard error) in the mean respiration rate when mixing the native species with *R. ponticum* at varying proportions (n = 7), calculated according to the equation described in the methods section. Briefly, values are positive for synergistic responses and negative for antagonistic responses, and the stronger the response, the greater the deviation from 0. No statistically significant differences ($P < 0.05$) in non-additive response strength were observed for neither of the three native species when comparing the mixtures with varying proportions of *R. ponticum* litter.

Species	**Litter proportion**	**Non-additive response strength**
A. pseudoplatanus	25%	0.526 $\pm$ 0.271
A. pseudoplatanus	50%	0.501 $\pm$ 0.328

A. pseudoplatanus	75%	0.138 ± 0.070
F. excelsior	25%	-0.035 ± 0.117
F. excelsior	50%	-0.282 ± 0.083
F. excelsior	75%	-0.147 ± 0.166
Q. petraea	25%	0.280 ± 0.217
Q. petraea	50%	0.516 ± 0.188
Q. petraea	75%	0.040 ± 0.124

3.4. R. ponticum leachate addition experiment

Leachates collected from microcosms containing *R. ponticum* litter were either left unaltered or treated with activated carbon which removed phenolics, before they were added to microcosms containing either *A. pseudoplatanus*, *F. excelsior* or *Q. petraea* litter. Overall, respiration in *F. excelsior* microcosms was significantly lower following the addition of unaltered leachate, relative to leachate with phenolics removed ($P = 0.035$) (Figure 5). This effect was not observed for microcosms containing *A. pseudoplatanus* or *Q. petraea* ($P = 0.116$ and $P = 0.094$ respectively). For all three species, respiration was significantly higher where phenolics were removed, compared to microcosms where distilled water was added ($P < 0.05$). However, there was no difference in respiration between unaltered leachate microcosms which included phenolics and distilled water ($P > 0.05$).

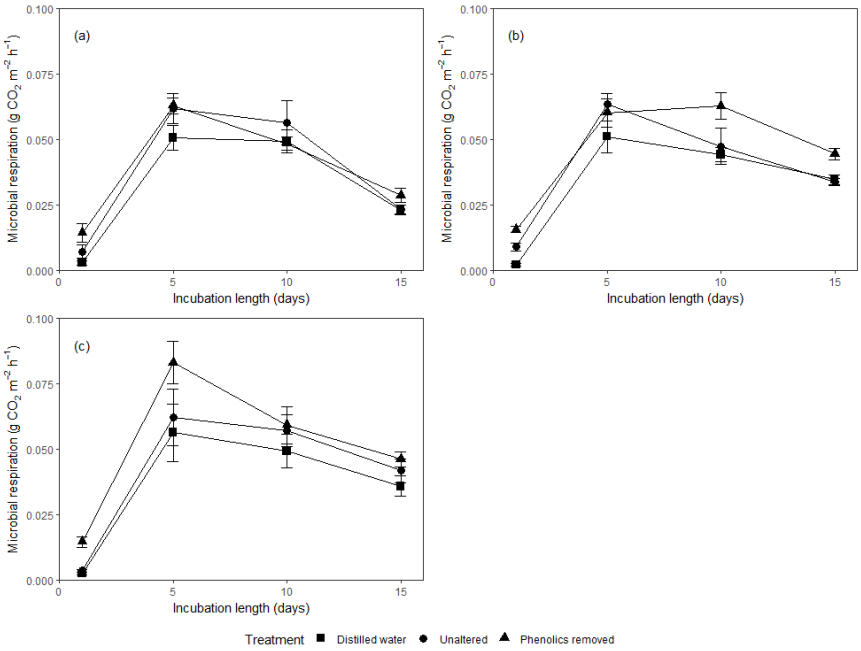

Figure 5: Mean microbial respiration (g CO_2 m^{-2} h^{-1}) (± standard error) over the course of the follow-up experiment, where leachate from decomposing *R. ponticum* litter were added to microcosms containing either *A. pseudoplatanus* (a), *F. excelsior* (b) or *Q. petraea* (c). There were three treatments; one where leachate was left unaltered, another where the leachate was treated with activated carbon to remove phenolics, and a distilled water control treatment (n = 7).

4. Discussion

This study focused on whether the litter chemical properties of invasive *R. ponticum* causes non-additive native tree litter decomposition. Results showed that *R. ponticum* has recalcitrant litter, with a high C:N ratio and phenolic compound content, decomposing slower than native labile litter (*A. pseudoplatanus* and *F. excelsior*) and at a similar rate to native recalcitrant litter (*Q. petraea*). When mixed with native litter, *R. ponticum* showed species-specific non-additive effects on decomposition. Non-additive microbial respiration was observed in 50% litter mixtures with *A. pseudoplatanus* and *F. excelsior*, in synergistic and antagonistic interactions respectively. No effect on microbial respiration was observed when mixed with these species at other proportions (25% or 75%), or when mixed with *Q. petraea*. The

366 proportion of *R. ponticum* mixed with native litter did not impact combined decomposition; there was
367 no difference in non-additive response strength when comparing the mix ratios containing different
368 proportions of *R. ponticum* for any of the three native species tested.

369 Litter chemical properties may explain the non-additive decomposition responses observed.
370 Antagonistic responses in microbial respiration were observed when *R. ponticum* litter was mixed with
371 *F. excelsior*, as hypothesised. *R. ponticum* litter had a higher C:N ratio than *F. excelsior*, which can cause
372 non-additive decomposition (Rosemond et al., 2010), whilst there was a significant negative correlation
373 between C:N and mean microbial respiration. This suggests that initial litter C:N may have contributed
374 towards the faster decomposition of *A. pseudoplatanus* and *F. excelsior* relative to *R. ponticum* and *Q.*
375 *petraea*, and the non-additive decomposition observed when mixing *R. ponticum* with *F. excelsior*.
376 Phenolic compounds leaching from litter can also influence decomposition by altering the decomposer
377 community (Fanin et al., 2014; Kuzyakov et al., 2000). Certain low-molecular weight phenolics stimulate
378 fungal spore germination and microbial growth, whilst more complex polyphenols such as condensed
379 tannins have a negative effect, inhibiting microbial activity (Hättenschwiler et al., 2005; Kuiters, 1990).
380 Phenolics leaching from *R. ponticum* litter may therefore have inhibited microbial activity, leading to
381 lower *F. excelsior* decomposition in mixed species microcosms.

382 The antagonistic decomposition of mixed *F. excelsior* and *R. ponticum* litter may also have been caused
383 by the formation of recalcitrant polyphenol-protein complexes (Hättenschwiler and Vitousek, 2000).
384 Tannins extracts from the related species *R. maximum* have a strong tendency to complex with
385 nitrogenous compounds (Wurzburger and Hendrick, 2007), including some enzymes, inhibiting
386 decomposition (Hättenschwiler and Vitousek, 2000; Horner et al., 1988; Palm and Sanchez, 1990). Few
387 organisms have the ability to degrade these complexes, with the exception of certain fungal species that
388 can synthesise polyphenol oxidase (Hättenschwiler and Vitousek, 2000; Kuiters, 1990). The nitrogen
389 content of *F. excelsior* litter was particularly high compared to the other three species, making microbial

activity in *F. excelsior* microcosms more likely to be affected by leaching polyphenols. Conversely, synergistic responses were observed when mixing *R. ponticum* with *A. pseudoplatanus*, whilst no effect was seen for *Q. petraea*. Both *Q. petraea* and *A. pseudoplatanus* litter had higher phenolic contents and C:N ratios than *F. excelsior*, potentially explaining why their decomposition was not suppressed when mixed with *R. ponticum*.

The importance of litter phenolic content in non-additive decomposition is supported by the results of the follow-up experiment, where leachates from decomposing *R. ponticum* litter were added to microcosms containing either *A. pseudoplatanus*, *F. excelsior* or *Q. petraea* litter. The addition of unaltered *R. ponticum* leachate, which contained 218 µg mL^{-1} of phenolics (Table 1S), suppressed microbial respiration in *F. excelsior* litter microcosms relative to leachate where >97% of phenolics had been removed with activated carbon. This suggests that phenolics released from decomposing *R. ponticum* were responsible for the antagonistic responses observed when mixed with *F. excelsior*. These results are supported by those of De Marco et al. (2018), who found that water extracts from *Robinia pseudoacacia* L. (black locust) and *Rubus fruticosus* L. (blackberry) litter reduced microbial activity and biomass when added to soil. Removing phenolics from the leachate had no effect for *A. pseudoplatanus* and *Q. petraea* microcosm respiration, potentially as they had higher phenolic contents than *F. excelsior* and were therefore less affected. This lack of effect for *A. pseudoplatanus* may partially explain why no antagonistic effect was observed when mixed with *R. ponticum* litter.

The strength of the observed non-additive effects did not increase with increasing *R. ponticum* proportions in litter mixes. This contrasts with the findings of Hickman et al. (2013), who suggested that the effect of invasive litter during the early phase of invasion is limited, with non-additive decomposition increasing in strength if invasion is allowed to progress. The effect of invasive litter on decomposition is likely to vary between species however; Elgersma and Ehrenfeld (2011) for example reported that small quantities of invasive *Berberis thunbergii* DC. (Japanese barberry) litter can cause substantial non-linear

414 shifts in decomposer communities. Our results could have important ecological implications, as they
415 suggest that even small quantities of *R. ponticum* litter can have cause profound changes in litter
416 decomposition for some native species.

417 Whilst significant non-additive responses in microbial respiration were observed for two of the three
418 native species when mixed with 50% *R. ponticum*, none of the six mixtures containing unequal
419 proportions of *R. ponticum* and native litter showed non-additive responses. This was unexpected, as
420 Mao and Zeng (2012) and Bonanomi et al. (2010) reported that having unequal proportions of litter led
421 to higher incidence of non-additive decomposition. In the current study, samples were milled and
422 incubated in darkness, whilst decomposition was monitored as microbial respiration and leached
423 carbon. Not separating the mass loss of different species' litters may mask small species-specific
424 decomposition responses (Hättenschwiler et al., 2005), potentially explaining why non-additive
425 responses were less common in the unequal mixtures. Despite this, we consider our approach to be
426 informative, as it allowed us to focus on the effect of litter chemistry, removing the effect of variations
427 in litter physical properties and photodegradation on decomposition. Photodegradation may not greatly
428 influence decomposition in the field, however, due to the dense shade imparted by the *R. ponticum*
429 canopy (Niinemets et al., 2003). Additionally, our approach allowed repeated measurements to be made
430 over time rather than at one timepoint, which is advantageous given that decomposition is a dynamic
431 and variable process (Hättenschwiler et al., 2005).

432 The dynamic nature of the decomposition process was reflected in the results of the current study, with
433 respiration declining over time for all species. This may be explained by soluble compounds leaching
434 from litter. At the start of the incubation, labile carbon sources would be readily leached from the litter
435 (Keuskamp et al., 2013), resulting in high microbial activity. Over time, the labile fraction of litter is
436 depleted, leaving behind the more recalcitrant structural compounds, resulting in a decreased
437 decomposition rate (Keuskamp et al., 2013). This was reflected in the decreasing leachate organic

carbon measurements observed over the incubation period in the current study, which were significantly correlated with the decreasing microbial respiration measurements. Under natural conditions, the effect of these compounds would be delayed and more prolonged, as the leaching of compounds from intact leaf litter would be slower due to lower litter surface area and temperature. In addition to the concentration, the composition of leachate carbon may also have an important influence on respiration. Microbial respiration in *R. ponticum* microcosms after two weeks was significantly lower than in *F. excelsior* microcosms, despite there being no difference in leachate total organic carbon concentration, possibly as *R. ponticum* litter was higher in inhibitory and recalcitrant phenolics. Litter phenolic content was negatively correlated with cumulative respired CO_2, suggesting that the high phenolic content of *R. ponticum* and *Q. petraea* contributed towards their slower decomposition rates relative to *A. pseudoplatanus* and *F. excelsior*.

Our results support observations made in the field of low nutrient turnover beneath *Rhododendron* spp. (Wurzburger and Hendrick, 2009, 2007), typical of ericaceous shrubs which are adapted for low-nutrient environments (DeLuca et al., 2013; Hobbie, 1992). Such plant-soil feedbacks are considered important drivers in the dominance of some plant species; litter inputs may change the soil's chemical properties, making it less favourable for species with different nutrient demands and more favourable for conspecifics (Van der Putten et al., 2013). *R. ponticum* may therefore promote its invasion and increase its dominance by altering the decomposition of native litter. Crucially however, we show that this effect on native litter decomposition was species-specific; *F. excelsior* and other native species with higher nutrient demands may be negatively influenced by altered soil conditions. Conversely, those with similar nutrient demands to *R. ponticum* may be less influenced by alterations in soil properties. These findings could be particularly important when restoring cleared sites to native habitats, as altered soil conditions influence the vegetation community that can establish post-clearance of *R. ponticum.*

5. Conclusions

This study highlights the strong influence of litter chemical composition on decomposition. Phenolic content, a group of compounds previously reported to inhibit decomposition, was particularly important, most likely explaining the slower decomposition of invasive *R. ponticum* litter relative to that of *A. pseudoplatanus* and *F. excelsior*, but not *Q. petraea*. Litter chemistry may also explain non-additive decomposition following litter mixing, with this effect varying between species. *F. excelsior* litter decomposition was slower than expected when mixed with *R. ponticum*. Conversely, combined decomposition for *A. pseudoplatanus* and *R. ponticum* was higher, whilst there was no effect for *Q. petraea*. The strength of the non-additive decomposition did not vary with increasing proportions of *R. ponticum* in litter mixtures. Following the removal of phenolics from *R. ponticum* litter leachates, microbial respiration was enhanced when added to microcosms containing *F. excelsior* litter, suggesting that these compounds may be responsible for antagonistic decomposition responses. This study highlights the potential for invasive shrubs to alter processes such as decomposition in plant-soil feedbacks, potentially shifting the natural balance of ecosystems. It also highlights that non-additive decomposition following invasive litter mixing is species-specific, being synergistic for some species and antagonistic for others.

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